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**FRITZ JOSEPH**

# GENETIC CHARACTERIZATION OF SURFACTANT-PRODUCING BACTERIA WITH LYSING ACTIVITY AGAINST *Pythium* ZOOSPORES IN HYDROPONIC LETTUCE

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> Dissertation presented to the Federal University of Lavras, as part of the requirements of the graduate program in Agronomy area Phytopathology, to obtain the master's degree.

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# ABSTRACT

*Pythium* root rot is one of the most severe diseases in hydroponic lettuce worldwide. Several species of *Pythium* may cause the disease, which is characterized mainly by root browning and poor plant growth. Application of chemical fungicides and disinfectants is the most common method used to manage these pathogens. Hydroponic systems offer an interesting opportunity to apply biological control agents or biologically derived products to control waterborne pathogens due to the controlled environment and the absence of soil. Despite the advantages of biological agents, products specifically developed to control *Pythium* in hydroponics are not common. Our goals in this study were to select, characterize and apply surfactant-producing bacteria to control *Pythium aphanidermatum* on hydroponically grown lettuce. From six bacterial strains initially known to produce surfactants, strain 88A secreted compounds with the highest activity. Genome sequencing of strain 88A and analyses of genomic indices revealed that it is a *Pseudomonas areruginosa*. A comparative genomic analysis of 309 genomes of this species showed that all genomes harbored genes *rhlA* and *rhlB* in one operon, which encode enzymes responsible for the synthesis of mono-rhamnolipids. An additional gene, *rhlC* located in another locus encodes for the conversion of mono- into di-rhamnolipids. Only one of these genomes had two copies of *rhlAB* and *rhlC* genes and seven genomes did not harbour the *rhlC* gene. The precipitated dry crude surfactants produced by strain 88A had properties that were similar to that of a mixture containing rhamnolipids, such as surface activity, foaming and capacity to lyse *Pythium* zoospores at concentrations equal and higher than 1 mg/ml. The identity of the rhamnolipid-encoding genes among 301 genomes of *P. aeruginosa* in relation to strain 88A varied from 98.9 to 99.9% for *rhlA*, from 98.7 to 100% for *rhlB* and from 97 to 100% for *rhlC*. The 303 sequenced strains deposited in databases and used in this study were isolated from animals (84.5%), plants (3.6%), soil (2.6%) and from environmental samples (9.2%). Strain 88A or the precipitated dry crude rhamnolipids decreased *Pythium* severity in hydroponic lettuce by approximately 60% and increased fresh weight of lettuce plants by 68%, when compared with the plants inoculated with *Pythium*. Although this bacterial species is frequently associated with immunocompromised patients, the purified rhamnolipids may be applied in the control of *Pythium* in hydroponic lettuce.

**Keywords**: *Lactuca sativa*, *Pseudomonas aeruginosa*, rhamnolipids, zoospores.

# RESUMO

A podridão da raiz de *Pythium* é uma das doenças mais graves da alface hidropônica em todo o mundo. Várias espécies de *Pythium* podem causar a doença, que se caracteriza principalmente pelo acastanhamento da raiz e pelo crescimento deficiente das plantas. A aplicação de fungicidas químicos e desinfetantes é o método mais comum usado para controlar estes patógenos. Os sistemas hidropônicos oferecem uma oportunidade interessante para aplicar agentes de controle biológico ou produtos derivados biologicamente para controlar patógenos transportados pela água devido ao ambiente controlado e à ausência de solo. Apesar das vantagens dos agentes biológicos, os produtos desenvolvidos especificamente para controlar o *Pythium* em hidropônicos não são comuns. Nossos objetivos neste estudo foram selecionar, caracterizar e aplicar bactérias produtoras de surfactantes para controlar o *Pythium aphanidermatum* em alface cultivada hidroponicamente. De seis estirpes bacterianas inicialmente conhecidas por produzir surfactantes, a estirpe 88A segregou compostos com a maior atividade. O sequenciamento genômico da estirpe 88A e análises de índices genômicos revelaram que se trata de uma *Pseudomonas areruginosa*. Uma análise genômica comparativa de 309 genomas desta espécie mostrou que todos os genomas abrigavam os genes *rhlA* e *rhlB* em um ópero, que codificam as enzimas responsáveis pela síntese de mono-rhamnolipídios. Um gene adicional, *rhlC* localizado em outro lócus encodes para a conversão de mono-rhamnolípidos em di-rhamnolípidos. Apenas um desses genomas tinha duas cópias dos genes *rhlAB* e *rhlC* e sete genomas não abrigavam o gene *rhlC*. Os surfactantes secos precipitados produzidos pela estirpe 88A tinham propriedades semelhantes às de uma mistura contendo ramnolípidos, tais como atividade superficial, formação de espuma e capacidade de lise de zoósporos *Pythium* em concentrações iguais e superiores a 1 mg/ml. A identidade dos genes codificadores de ramnolipídios entre 301 genomas de *P. aeruginosa* em relação à cepa 88A variou de 98,9 a 99,9% para *rhlA,* de 98,7 a 100% para *rhlB* e de 97 a 100% para *rhlC*. As 303 cepas sequenciadas depositadas em bancos de dados e utilizadas neste estudo foram isoladas de animais (84,5%), plantas (3,6%), solo (2,6%) e de amostras ambientais (9,2%). A cepa 88A ou os ramnolípidos secos precipitados diminuiu a severidade do *Pythium* em alface em aproximadamente 60% e aumentou o peso fresco das plantas em 68%, quando comparado com as plantas inoculadas com *Pythium*. Embora esta espécie bacteriana esteja frequentemente associada a pacientes imunocomprometidos, os ramnolípidos purificados podem ser aplicados no controle do *Pythium* em alface hidropônica.

**Palavras-chave**: *Lactuca sativa*, *Pseudomonas aeruginosa*, rhamnolipídeos, zoósporos.

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# 1. INTRODUCTION

*Pythium* zoospores released from sporangia are the primary source of inoculum in hydroponics. Managing this asexual stage reduces the damage caused by this pathogen (ISLAM; LAATSCH; TIEDEMANN, 2016). Chemical disinfectants are commonly used to manage *Pythium* (BAGNALL, 2008; GODDEK, 2019; STANGHELLINI, 1984). Hence, approved chemicals are very limited (COOLONG, 2012; TESORIERO; FORSYTH, 2011), very expensive, phytotoxic and harmful to the environment (BAGNALL, 2008; CROUZET et al., 2020). The non-selective control methods used to disinfect the nutrient solution, such as heat, UV radiation and ozone (GODDEK, 2019; SON; KIM; AHN, 2015) have the disadvantage of eliminating both beneficial and harmful microorganisms (GODDEK, 2019; MONARCA et al., 2000). As the use of chemicals and active treatments in hydroponics are undesirable, the use of organisms that can compete with the pathogen by generating antibiotic compounds have been explored (BERG et al., 2017; ZOHARA et al., 2016). In the course of biological control strategies, it was found that surfactant-producing bacteria and their derived products can be implemented as a microbiome-based solution (PUPIN et al., 2018; TANAKA et al., 2015). Among these, rhamnolipids, which consists of a carbohydrate moiety linked to fatty acids, are among the most studied biosurfactants (BANAT, 1997; INÈS; DHOUHA, 2015; THAKUR et al., 2021). These extracellular biomolecules are synthesized by genes directed by quorum sensing (CHONG; LI, 2017). Genes *rhlAB* in one operon, encode rhamnosyltransferase I essential for the synthesis of β-Hydroxydecanoyl-β-Hydroxydecanoate (HAA) and mono-rhamnolipids. The *rhlC* gene encodes rhamnosyltransferase II, which adds another rhamnosyl group to convert mono into dirhamnolipid (OCHSNER; HEMBACH; FIECHTER, 1996; THAKUR et al., 2021; ZHU; ROCK, 2008).

*Pseudomonas aeruginosa* is recognized as the ultimate producer of rhamnolipids (CHONG; LI, 2017; EL-HOUSSEINY et al., 2020; GIBSON et al., 2010; THAKUR et al., 2021). Due to their lack of toxicity, high biodegradability, capacity to lower the surface tension and inhibitory activity at low concentrations, these biomolecules have gained attention in agriculture (PUPIN et al., 2018; SACHDEV; CAMEOTRA, 2013; TANAKA et al., 2015). Therefore, these biomolecules play a primary role in the disruption of plasma membranes (ISLAM; LAATSCH; TIEDEMANN, 2016; SHARMA et al., 2007). Sharma et al. (2007) have demonstrated the involvement of rhamnolipids in zoospore inhibition, zoospore lysis, and inhibition of the hyphal growth of a wide range of oomycetes. De Jonghe

et al. (2005) reported strains of *Pseudomonas aeruginosa* producing rhamnolipids which suppressed the spread of brown root rot caused by *Phytophthora cryptogea* in witloof chicory (*Cichorium intybus* var. *foliosum* ) grown in hydroponics. *Pseudomonas aeruginisa* strain KVD-HM52 has been reported to produce mono and di-rhamnolipids with potent antifungal activity, which has been shown to control *Fusarium oxysporum* wilt disease in tomato plants (DEEPIKA; SRIDHAR; BRAMHACHARI, 2015). Surfactant-producing bacteria may also promote plant growth. The rhomnolipids produced by *Pseudomonas putida* BSP9 isolated from the rhizosphere resulted in improved growth of *Brassica juncea* (MISHRA et al., 2020). Likewise, *Pseudomonas aeruginosa* RTE4 strain isolated  from rhizosphere soil of tea produces rhamnolipids which have been shown to be effective against the leaf fungi *Corticium invisium* and *Fusarium solani*, and as a result promoted plant growth (CHOPRA et al., 2020). Indeed, surfactant-producing bacteria and their by-products exhibited effectiveness in controlling zoospore-producing microorganisms, as well as promoting plant growth. Owing to its role in inhibiting zoospores activities, its use in the control of *Pythium* in lettuce grown in hydroponics may be desirable.

Thus, this study aimed to select, characterize and apply surfactant-producing bacteria to control *Pythium* on hydroponically grown lettuce. For this, the following hypotheses are proposed:

1.  The bacterial collection assessed during these studies contains strains producing antagonistic biosurfactants, which lyse *Pythium* zoospores;
2.  The genes that encode for the synthesis of these compounds will be found in the genome of these strains;
3.  The bacterial collection contains strains producing biosurfactants that will be able to reduce *Pythium* disease in hydroponic lettuce.

## 2. BACKGROUND

### 2.1. Surfactants

Surfactants are secondary metabolites composed of a hydrophobic fatty acid tail linked to a hydrophilic peptide fragment (CROUZET et al., 2020; PUPIN et al., 2018; SACHDEV; CAMEOTRA, 2013). This amphiphilic structure explains their properties to reduce the surface and interfacial tension of a fluid (NAYARISSERI; SINGH; SINGH, 2018). Surfactants can be of chemical or biological origin and are generally classified according to their chemical charge and their molecular weight. Considering their chemical charge, these surface-active agents are classified in anionic, non-anionic, cationic and zwitterionic (NAYARISSERI; SINGH; SINGH, 2018; SAŁEK; GUTIERREZ, 2016). Based on the molecular weight, the surfactant form two main groups: low molecular weight, surface-active agents that have the ability to reduce the surface tension of two immiscible fluids, and the high molecular weight that are able to act as emulsifiers (SAŁEK; GUTIERREZ, 2016), thus facilitating the formation of water-in-oil (W/O) and oil-in-water (O/W) emulsions (SADAT SADATRASUL et al., 2017). These surfactants are commonly used in food and pharmaceutical industries. Surfactants can be obtained from biological sources (plants, animals and microorganisms). Many microorganisms associated with the rhizosphere produce biosurfactants (NAYARISSERI; SINGH; SINGH, 2018). These biomolecules govern many biological reactions such as plant-pathogen interactions (CROUZET et al., 2020; NATHOO et al., 2017; NAYARISSERI; SINGH; SINGH, 2018; SACHDEV; CAMEOTRA, 2013; TRAN; KRUIJT; RAAIJMAKERS, 2008).

### 2.2. Surfactants and biological control

Biosurfactants may be used as an alternative for controlling pathogens and to promote a sustainable and environmentally friendly agriculture through the reduction or total elimination of traditional chemical pesticides (CROUZET et al., 2020; SHALINI et al., 2017). These biomolecules can be up to 76.9% effective in plant protection (SHALINI et al., 2017). Biosurfactants are effective in zoospore inhibition, zoospore lysis and inhibition of hyphal growth of a wide range of pathogens (CROUZET et al., 2020; SHARMA et al., 2007). Their major role in inhibiting zoospores may attract interest in their use to control zoosporogenic microorganisms in soilless culture systems. Nielsen et al. (2002) have demonstrated the antagonistic properties of the cyclic lipopeptides type biosurfactant

affiliated with *Pseudomonas fluorescens* strain against root pathogenic *Pythium ultimun*. The involvement of rhamnolipid biosurfactants in lysis of plasma membrane of *Pythium* and *Phytophtora* zoospores has been demonstrated by Sharma *et* al. (2007). *Pseudomonas fluorescens* strains have been reported to reduce *Pythium* root rot caused by *Pythium aphadermatum* in a rock-wool cucumber soilless system (MCCULLAGH et al., 1996). De Souza et al. (2003) isolated from rhizosphere of wheat, the bacterial strain *Pseudomonas fluorescens* SS101, which produces the cyclic lipopeptide massetolide, capable of inhibiting the motility of zoospores and induced the lysis of pathogenic microorganisms such as *Phythium ultimum* var. *sporangiiferum*. Liopopeptides produced by *Bacillus subtilis* SPB1 train showed high antifungal activities against *Fusarium solani*. Biosurfactants reduced the spread of the dry root potato tuber and symptom manifestation by 78 and 100%, respectively (MNIF et al., 2015).

## 2.3. Bacteria producing surfactants

Many bacterial strains produce biosurfactants. The biosurfactants are more efficient, selective, environmentally friendly, and stable than synthetic surfactants (BANAT, 2014; PUPIN et al., 2018). The widely known biosurfactant are glycolipids, lipopeptides, lipoproteins, and heteropolysaccharides (BANAT, 1997; NAYARISSERI; SINGH; SINGH, 2018). The table below presents a list of bacteria that produce biosurfactants.

Table 1- Reports of biosurfactant derived from bacteria with potential in agriculture.

| Species | Type of biosurfactant | References |
|---|---|---|
| *Serratia rubidaea* **SNAU02** | Rhamnolipids | Nalini and Parthasarathi, 2018 |
| *Bacillus subtilis* | Lipopeptides | Pupin *et* al., 2018 |
| *Pseudomonas* **sp.** | Rhamnolipids | Sharma *et* al.,2007 |
| *Acinetobacter* **sp.** | Glycolipids | Shalini *et* al., 2017 |
| *Pseudomonas aeruginosa* | Rhamnolipids | Benincasa et al., 2004 |
| *Pseudomonas fluorescens* | Lipopeptides | De Souza et al., 2003 |
| *Pseudomonas putida* | Lipopeptides | Kuiper et al., 2004 |
| *Pseudomonas fluorescens* | Viscosin | De Bruijn and Raaijmakers, 2009 |
| *Bacillus subtilis*, *Bacillus pumilus* | Surfactin | Ghelardy et al. 2012 |
| *Bacillus polymyxa* | Polymixins | Falagas and Kasiakou,2005 |
| *Serretia marcescens* | Serrawettin | Li et al., 2005 |
| *Pseudomonas fluorescens* | *N*-acetyl & *O*-pyruvil heteropolysaccharide | Bonilla et al., 2005 |
| *Bacillus subtilis* | Fengycin | Zhang and Sun, 2018 |
| *Bacillus* **sp.** | Iturin | Zhou et al. 2020 |

Glycolipids are the most common biosurfactants, in which carbohydrates compounds like glucose, mannose, galactose, trehalose, rhamnose, sophorose are attached to a long-chain aliphatic acid (BANAT, 1997; NAYARISSERI; SINGH; SINGH, 2018). Glycolipids have been shown to be very effective against various microorganisms such bacteria, viruses and fungi due to their powerful role in destabilizing biological membranes (INÈS; DHOUHA, 2015; SHARMA et al., 2007). Glycolipids are subdivided into rhamnolipids, trehalolipids, sophorolipids among others (INÈS; DHOUHA, 2015). Rhamnolipids exhibited a wide range of properties such as the ability to reduce the surface tension and interfacial tension (36 mN/m), high emulsifying power (63%), foaming potency, as well as their high production in very short time period (EL-HOUSSEINY et al., 2020; INÈS; DHOUHA, 2015). *Pseudomonas aeruginisa* is the most predominant species for production of rhamnolipids. Rhamnolipids are composed of one or two L-rhamnose sugars, linked together through α-1,2-glycosidic linkage and one or more saturated/unsaturated β-hydroxy fatty acids chains (THAKUR et al., 2021).

## 2.4. Synthesis of rhamnolipids

Rhamnolipid production is heavily dependent on the culture medium and the growth phase. Numerous studies have been carried to find a medium capable of stimulating the synthesis of rhamnolipids from basic precursors. Nutrient broth was one of the first media attempted, however, it provided low surfactant yields. Minimal salts medium appears to give higher yields. Among these, Cooper's medium have been defined, containing $NH_4NO_3$ as the nitrogen source and glucose as carbon source (COOPER; GOLDENBERG, 1987). The rhamnolipid biosynthesis pathway requires the *rhlA*, *rhlB* and *rhlC* genes and three mains steps. In the first step, *rhlA* encodes an acyltransferase, which converts the hydroxyacyl-acyl carrier protein intermediates into the synthesis of fatty acids in 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) component of rhamnolipids (TAN; LI, 2018; WITTGENS et al., 2017; ZHU; ROCK, 2008). While, the sugar moiety-deoxythymidine diphosphate (dTDP)-L-rhamnose precursor of rhamnolipids is synthesized from glucose during an enzymatic process using enzymes such as glucose-1-phosphate thymidyltransferase (RmlA), dTDP-d-glucose-4,6-dehydratase (RmlB), dTDP-4-keto-6-deoxy-d-glucose-3,5-epimerase (RmlC), and dTDP-4-keto-l-rhamnose reductase (RmlD). In the second step, *rhlB* that encodes a rhamnosyltransferase condenses 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) and (dTDP)-L-rhamnose to form mono-rhamnolipid. In the final step, *RhlC* rhamnosyltransferase

using another molecule (dTDP)-L-rhamnose to convert the resulting mono-rhamnolipid into di-rhamnolipid (WITTGENS et al., 2017; ZHU; ROCK, 2008) (figure.1).
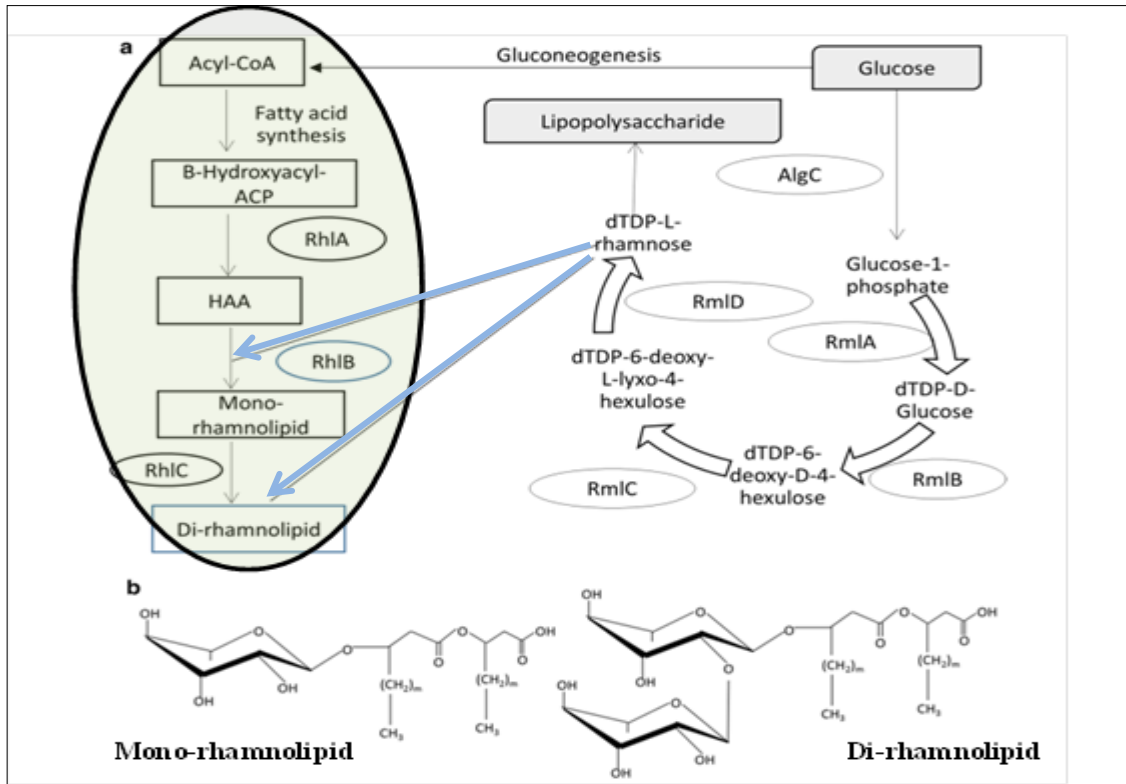


Figure 1- Biosynthesis of rhamnolipids in *Pseudomonas*. a) Pathway of rhamnolipids synthesis, and metabolic pathway of glucose and Acil-coA synthesis b) chemical structure of mono & di-rhamnolipids.

Source: Lee et al. (2018).

## 2.5. *Pythium*

*Pythium* belongs to the family *Pythiaceae* of the Oomycete class. This class has been considered as fungi for approximately two centuries. Nowadays the Oomycetes class is described as fungal-like in the kingdom Straminipila (Chromista) (LÜCKING et al., 2021). *Pythium* species have filamentous sporangia, smooth-walled spherical oogonia and stalked antheridia. Several species are highly damaging to plants, which are usually responsible for damping off and rots. These microorganisms produce motile zoospores that easily propagate through water, which makes *Pythium* one of the most prevalent pathogens in hydroponics (CHOUDHARY et al., 2016; GODDEK, 2019). In addition to zoospores, *Pythium* infection can occur through sporangia and mycelia (BAGNALL, 2008; BURGOS, 2013; MOULIN; LEMANCEAU; ALABOUVETTE, 1994). According to Gold (1985), *Pythium* needs just 5

minutes after inoculation to penetrate the plant roots. The process includes zoospore encystment at the root surface, synthesis of a thick cell wall, adhesion to the root surface,
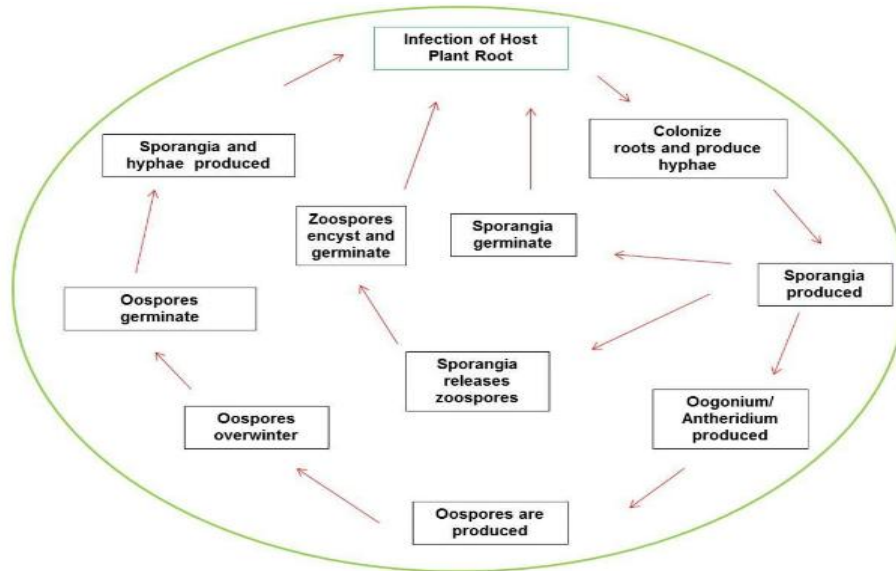


Figure 2- Disease cycle of typical *Pythium* diseases.

Source: Bourgos-Garay, (2013).

formation of germ tubes or appressoria-forming hyphae and penetration of the root surface (HARDHAM, 2007; LAROUSSE; GALIANA, 2017).

The main symptoms resulting from the attack of *Pythium* in hydroponic systems are browning (*P. aphanidermatum*) or extensive yellowing (*P. dissotocum*), architectural changes (with all species), root bulge (*P. dissotocum*) and cell callus proliferation (*P. dissotocum*) (OWEN-GOING; SUTTON; GRODZINSKI, 2003). Figure 1 shows the cycle of typical *Pythium* diseases. The process involves the inoculation, colonization, and proliferation of *Pythium* in host plant tissues.

The most prevalent *Pythium* species in hydroponic systems are *Phytium aphanidermatum, Pythium dissotocum, Pythium ultimum* var *ultimum*, and members of group F (absence of the sexual phase) (MOULIN; LEMANCEAU; ALABOUVETTE, 1994; STANGHELLINI, 1984). *Pythium aphanidermatum, P. dissotocum* and group F *Pythium* produce zoospores in large numbers in sporangia (GOLD, 1985; SUTTON et al., 2006) *P. ultimum* var. *ultimum* does not usually form sporangia and zoospores are produced rarely (Van Der Plaats-Niterink 1981). *Pythium aphanidermatum* is characterized by the possession

of swollen sporangia. *Pythium dissotocum* has filamentous sporangia branched, not or slightly inflated. The group F species are characterized by non-inflated filamentous sporangia (Van Der Plaats-Niterink, 1981). Some other species such as *P. irregulare*, *P. coloratum, P. sulcatum, P. diclinum* and *P. myriotylum* can cause root diseases in hydroponic

Table 2- Characteristics of reproductive organs of *Pythium* species.

| Species | Characteristics | | | |
|---|---|---|---|---|
| | **Sporangia** | **Oogonia** | **Antheridia** | **Oospores** |
| *P. aphanidermatum* | Inflated filamentous | Terminal, globose | Monoclinous or diclinous | Aplerotic |
| *P. dissotocum* | Filamentous | subglobose | Mono or diclinous | Aplerotic or nearly plerotic |
| *P. group F* | Filamentous non-swollen | Not formed | Not formed | Not formed |
| *P. irregulare* | Seldom formed | Globose to irregular | Monoclinous | Aplerotic |
| *P. sulcatum* | Filamentous | globose, smooth | monoclinous and diclinous | Aplerotic |
| *P. diclinum* | Filamentous non-inflated | spherical or ovoid | Diclinous | Aplerotic |
| *P. myriotylum* | Filamentous | Subglobose | Diclinous | Aplerotic |
| *P. ultimum var ultimum* | Not formed | Globose | Monoclinous | Aplerotic |

Source**:** Van Der Plaats-Niterink (1981).

systems (TESORIERO; FORSYTH, 2011). The sexual reproduction of *Pythium* species takes place by means of oogonia and antheridia. Oogonia are spherical to limoniform and are intercalary or terminal. The male organs, the antheridia are termed monoclinous if they originate from the oogonial stalk and diclinous if they originate from a different hypha not closely connected with the one subtending the oogonium. The oospores can fill the whole oogonium (plerotic oospores) or leave some space between the oogonial and oospore wall (aplerotic oospores) (DA SILVA et al., 2019; LÉVESQUE; DE COCK, 2004; STAMPS, 1982). The characteristics of reproductive organs of different species are summarized in the table 2.

## 2.6. Control of *Pythium* in hydroponic systems

*Pythium* root rot is a worldwide concern and there is a continuous impact on the plant growth in hydroponics. The traditional method of controlling root rot is the use of

disinfectants (GOLD, 1985; STANGHELLINI, 1984; TESORIERO; FORSYTH, 2011). Other control methods include the use of heat, UV radiation and ozone (GODDEK, 2019; GOLD, 1985; MONARCA et al., 2000). Alternative control strategies with the use of biocontrol agents such as surfactant-producing organisms (CROUZET et al., 2020; MNIF et al., 2015; MORUZZI et al., 2017; SHARMA et al., 2007), among others, are used to control waterborne pathogen in hydroponic systems.

## 2.6.1. Chemical control

Stanghellini (1984) observed a reduction in spinach root rot when he applied 10 μg of Metalaxyl per milliliter of nutrient solution. It has been shown that the use of 5 μL of Metalaxyl per liter of nutrient solution inhibited the production of zoospores of *P. aphanidermatum* and *P. dissocotum*. The chemical product reduced root rot, as well as increased spinach yield (GOLD, 1985). In Australia, the application of 2.5 μL / L of Acibenzolar-S-methyl in nutrient solution have reduced coriander root rot caused by *P. sulcatum* but a slight phytotoxicity has been observed (TESORIERO; FORSYTH, 2011). The application of chitosan at a concentration of 100 or 400 μg per liter of nutrient solution significantly reduced cucumber root rot caused by *P. aphanidermatum* and triggered several host defense responses, including the induction of structural barriers in root tissues and stimulation of antifungal hydrolases (chitinase, chitosanase and β-1,3-glucanase) in roots and leaves (EL GHAOUTH et al., 1994). Oxidants such as ozone, chloride are commonly used to disinfect the nutrient solution. A decrease of onion root rot was observed after wastewater disinfestations with ozone (MONARCA et al., 2000). Despite the effectiveness of chemical disinfectants in controlling *Pythium*, their performance as control strategy can be detrimental. Bagnall (2008) observed a significant reduction of viable inoculum after minutes of exposure of *Pythium* zoospores in an aqueous suspension containing the disinfectants Actsol, Agral 90, Fitosan, Prasin, Purogene, TecsaClor, Sporekill and copper sulfate. However, the reduction in inoculum rate did not involve an increase in lettuce yield, due to stunting caused by pesticides toxicity (BAGNALL, 2008). Oxidation may also be harmful for the whole resident microflora, as well phytotoxic and there are still questions whether the oxidants enter the root zone (EHRET et al., 2001).

## 2.6.2. Physical control

Temperature is an important factor in the development of root rot in hydroponic systems. Temperature and light conditions influence the onset of symptoms (OWEN-GOING; SUTTON; GRODZINSKI, 2003). In 1985, Gold tested different temperatures on the virulence of *P. aphanidermatum* and *P. dissocotum* and noted that zoospore production and their penetration into root tissues is favored by temperatures between 17 and 30℃. Both species caused a decline of spinach yield at 21, 27℃ and 30℃. However, *P. dissocotum* was able to reduce yield at 17℃. An abundant zoospore production was observed at 30℃ (GOLD, 1985). Disinfection with heat, filters and UV radiation are used to minimize the risk of spreading water-borne pathogens in hydroponics (EHRET et al., 2001; SON; KIM; AHN, 2015). Stanghellini et al. (1986) obtained a significant reduction of spinach root rot caused by *P. aphanidermatum*, when the nutrient solution infested with zoospores and encysted zoospore was submitted to UV light treatment. The use of heat for disinfecting the nutrient solution is the most reliable method to eradicate the pathogen. Each group of microorganism has its specific lethal temperature (GODDEK, 2019). Nevertheless, it is important to emphasize that the great diversity of microorganisms decreases at temperature above 70℃ (STEINBERG et al., 1994). This lower diversity and density of microorganisms may leave the plants more vulnerable to root rots.

## 2.6.3. Biological control

Biological control could be very effective in hydroponic systems. Under field conditions, it is difficult to control the environmental parameters that often have adverse effects on the adaptation of biological control agents. Conversely, in greenhouse, temperature, pH and humidity can be adjusted to offer the control agents ideal growth conditions (CORNELIS et al., 2012; MHADHBI, 2012; NGUYEN; MCINTURF; MENDOZA-CÓZATL, 2016). Moreover, in hydroponic systems the substrate is virtually sterile, and the control agents can easily establish onto the substrate (PAULITZ; ZHOU; RANKIN, 1992). Early studies revealed the advantages of using biocontrol agents in soilless systems. These microorganisms use different mechanisms such as antibiotic production, root colonization, competition for space and nutrients and induced systemic resistance (ISR) to control target pathogens. Paulitz et al. (1992) succeeded to inhibit mycelial growth and zoospore germination of *Pythium aphanidermatum* with bacteria isolated from the rhizosphere of cucumber. *Enterobacter cloacae* has been reported to suppress the mycelial

growth of *P. aphanidermatum*, as well as to reduce *Pythium* damping off of cucumber by up to 68% (KAZEROONI et al., 2020). Likewise, *Paenibacillus polymyxa* (isolate 4) displayed a strong inhibitory activity against *Pythium helicoides* root rot in hydroponic lettuce (*Lactuca sativa var.* Crispa). Additionally, the treatment with the control agent resulted in a significant increase in the growth of lettuce plants (ADHIKARI, 2019). In recent years, the role of biosurfactants in controlling pathogenic fungi and oomycetes have been elucidated (MNIF et al., 2015; PUPIN et al., 2018; SACHDEV; CAMEOTRA, 2013; SHALINI et al., 2017; TRAN; KRUIJT; RAAIJMAKERS, 2008).

## 2.7. Hydroponic systems

Hydroponic cultivation is technically defined as a soilless method for growing plants using a mineral nutrient medium (MHADHBI, 2012). Hydroponics is used worldwide to produce foliage, flowers and vegetable crops (SON; KIM; AHN, 2015). The six main types of soilless cropping systems are deep water culture, nutrient film technique (NFT), wick, ebb and flow, drip system (LEE; LEE, 2015; SHARMA et al., 2018; SON; KIM; AHN, 2015; WANG et al., 2008). The NFT and the drip system are the most widely used for both research and commercial production (MONTEIRO et al., 2012; SON; KIM; AHN, 2015). In Latin America, particularly Brazil, the country with the largest area of soilless cultivation in the region, the drip system and NFT are the most commonly used (MONTEIRO et al., 2012; RODRÍGUEZ-DELFÍN, 2012). Hydroponic systems can be grouped into two categories, the liquid hydroponic system in which the roots of plants grow only in the  nutrient solution and the aggregate hydroponic system when the roots of the plants develop on inert substrates such as sand, gravel, peat, perlite, vermiculite, rock wool, among others (CORNELIS et al., 2012; MHADHBI, 2012). According to the water supply, hydroponic systems may be closed or open. The system is closed when the nutrient solution is recirculated, while in the open system, the nutrient solution is not recirculated, which can flow freely as wastewater or can be used for other purposes (CORNELIS et al., 2012; MHADHBI, 2012).

Hydroponic systems guarantee the availability of essential elements in the plant growth environment (GIL-MONREAL et al., 2018). According to Nguyen and others, in soilless cropping systems the nutrient concentration of the solution can be easily adjusted, allowing plants to respond to a deficiency or excess of essential elements (MHADHBI, 2012; NGUYEN; MCINTURF; MENDOZA-CÓZATL, 2016). Therefore, the hydroponic cropping system allows to understand the relationship between nutritional status and plant

development (CORNELIS et al., 2012; MHADHBI, 2012). Soilless cultivation systems are getting a lot of attention nowadays due to the high automation and technology available. The soilless cropping system is an efficient way to produce crops of high nutritional value and high yield per unit of area (MONTEIRO et al., 2012; TORABI; MOKHTARZADEH; MAHLOOJI, 2012). The decrease in agricultural soils in the world may increase the interest in the use of hydroponic systems. In Latin America, the area of soilless cultivation is increasing and there is great interest in learning this production technique (RODRÍGUEZ-DELFÍN, 2012). The main species cultivated in hydroponics are cucumber (*Cucumis sativus* L.), lettuce (*Lectuca sativa* L), tomatoes (*Lycopercicum esculentum* Mill), spinach (*Spinacia oleracea* L) and pepper (*Capsicum annuum* L.) (ADHIKARI, 2019; SHARMA et al., 2018; SON; KIM; AHN, 2015). Lettuce is one of most popular hydroponic crops (COOLONG, 2012; GENUNCIO et al., 2012). The production of lettuce in hydroponics is of prime importance as lettuce is easy to grow and maintain (COOLONG, 2012). The lettuce life cycle in hydroponic is shorter when compared to the traditional cultivation. A maximum of 35-45 days are needed to harvest lettuce in hydroponics (SHARMA et al., 2018). In Brazil, lettuce is cropped mainly in NFT whereas the drip irrigation system is used exclusively for fruit vegetables (MONTEIRO et al., 2012; RODRÍGUEZ-DELFÍN, 2012). A great diversity of inorganic and/or organic substrates is used in hydroponics (GODDEK, 2019; MONTEIRO et al., 2012; RODRÍGUEZ-DELFÍN, 2012). The soilless system presents a great concern associated with the easy spread of root pathogens, as a single diseased plant can contaminate the entire crop (AMALRAJ; TAYLOR; SUTTON, 2019; COOLONG, 2012; SON; KIM; AHN, 2015). Among them, zoosporic pathogens such as *Phytophtora* and *Pythium* can easily propagate in the aqueous medium causing serious damage and result in significant losses (CHOUDHARY et al., 2016; COOLONG, 2012; MATHIAS, 2009).

## 3. MATERIAL AND METHODS

### 3.1. Microorganisms and growth condition

*Pythium* was isolated from soil recovered in the Floriculture sector at the Federal University of Lavras (UFLA). Soil samples were transferred to a closed plastic box. Subsequently, pieces of cucumber (5mm in thickness) which had been cut horizontally were placed on the soil surface and kept moist at room temperature for 48h until the white mycelium appeared. After incubation, coenocytic hyphae plugs were aseptically transferred onto Potato Dextrose Agar (PDA) plates (200g/l potatoes, 20g/l dextrose and 20g/l agar) for purification. The plates were incubated at 25 °C for 72h. After this growth period, the Petri plates were kept at room temperature for further analysis.

Four bacterial strains *Pseudomonas aeruginosa* (88A, LBB-47, LBB-58) and *Bacillus subtillis* LBB50 were obtained from an experimental collection at Federal University of Vicoça and two strains of *Bacillus subtillis (*BMH and BINV*)* were obtained from the laboratory of Molecular Phytopathology at UFLA. The *Pseudomonas* strains were cultured on king medium B (agar, 15g; meat peptone, 20; $KH_2PO_4$, 1.5, glycerol, 5mL and 1000mL of distilled water, amended with 30μg/mL of chloramphenicol) plates, while *Bacillus* were plated on nutrient-agar (agar, 20g; bacteriological peptone, 5 g; beef extract, 3g and 1000mL of distilled water), and incubated for 24h at 37 °C and 30 ± 2 °C respectively. For short-term use, the plates were stored at room temperature.

### 3.2*. Pythium* identification

#### 3.2.1. DNA extraction

*Pythium* isolates were cultured on PDA plate and incubated at 28 ◦C for 4 d. Then, a small piece of coenocytic hyphae grown on the edge of the plate was transferred into 250 mL Erlenmeyer flask containing 150 mL Potato dextrose broth (200g/l potatoes, 20g/l dextrose) and was incubated at room temperature for 7 d. The mycelium was collected by filtration trough sterile filter paper, dried at room temperature for 2 d under a laminar flow hood. To extract the DNA, 1 g of dried mycelium was ground to a fine powder with a mortar and pestle in liquid nitrogen. The powder was transferred to microcentrifuge tubes (2mL) and mixed 1 mL of 10% CTAB extraction buffer (10mM tris base (pH 8.0), 20mM EDTA (pH 8.0), 1.4 M NaCl, CTAB(10%), mercaptoetanol (0.1%) and PVP(0.2%), vortexed for 1 min and incubated at 65 ◦C for 1h. An equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1,

v: v: v) mixture was added to the suspension. The mixture was vortexed for 1min, followed by centrifugation at 11000 rpm for 10 min. Samples were transferred to a new tube (2 mL) and an equal volume of isoamyl-alcohol (24:1, v: v) was added, the suspension was vortexed for 1min and was centrifuged at 11000 rpm for 10min. The supernatant was transferred to a new clean tube (1.5mL) and mixed with 1mL of Isopropanol. The suspension was incubated at -74 for 1h to precipitate the DNA. The precipitate was collected by centrifugation, the supernatant was discarded, and the pellet was washed twice with 70% ethanol. The precipitate was centrifuged again at 11000rpm for 10min and air-dried. The pellet was suspended in 50 µl TE buffer [10 mM Tris-HCl (pH 8), 0.1 mM EDTA (pH 8)], and the DNA concentration was estimated using a Nano-drop spectrophotometer.

### 3.2.2. PCR amplification and sequencing

The mitochondrial c oxidase subunit 2 (cox2) and internal transcribed spacer (ITS) regions were amplified by PCR. To amplify the cox2 gene, the forward primer cox2-FM66 (5′-TAGGATTTCAAGATCCTGC-3′) and the reverse primer *cox*2-FM58 (5'-CCACAAATTTCACTACATT-3') were used (VILLA et al., 2006). To target the ITS region, the forward universal eukaryotic primer UN-up18S42 (5′-CGTAACAAGGTTTCC-3′) and the reverse primer, UN-LO28S22 (5'-GTTTCTTTTCCTCCGCTTATTGATATG-3′) (LÉVESQUE; DE COCK, 2004) were used. The PCR reactions contained 1 µl of DNA (30 ng µl$^{-1}$), 5 µl reaction buffer, 2.5 µl MgCl$_2$ (25 mM), 0.5 µl (10µM) of each primer, 0.5 µl dNTPs (10mM) and 0.3 µl Taq DNA polymerase and 14.7 µl deionized of purified water in a total volume of 34 µl. The amplification was carried out with PCR condition of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C, and a final extension of 72 °C for 7 min. PCR fragments were purified after agarose gel electrophoresis. The presence of bands in the agarose gel was detected under UV light. PCR products were subjected to DNA sequencing with the Sanger method to identify the strains. The sequences obtained for isplates UFLA1 and UFLA2 were compared with the ones deposited in public databases.

### 3.3. Pathogenicity of *Pythium*

Lettuce (*Lactuca sativa* cv. Roxa) seedlings with 4 definitive leaves obtained from a commercial nursery were used to assess the pathogenicity of two different *Pythium* strains. Seedling roots were washed with sterile distilled water to remove all the substrate and subsequently transferred to 300mL cups filled with 140 mL of nutrient solution. The lettuce

seedlings were supported by a smaller plastic cup and the experiment was kept at room temperature for 48h to allow the plant roots to adapt to hydroponic growing conditions before the inoculation with *Pythium*. The nutrient solution was composed of the fertilizer doses of 540 mgL$^{-1}$ calcium nitrate [Ca(NO3)$_2$] and 720 mgL$^{-1}$ of Maxsol-MX21 [nitrogen (N), 8%; phosphorus (P2O5), 11%; potassium (K), 38%; magnesium (Mg), 1.6%; sulfur (S), 2.9%; iron (Fe), 0.2%; zinc (Zn), 0.02%, manganese (Mn), 0.04%; copper (Cu), 0.004%; boron (B), 0.02%; molybdenum (Mb), 0.004% ]. Each compound was previously dissolved in an equal volume of water, and then autoclaved at 121 ∘C for 30 min before mixing to avoid precipitation. After the initial 2 d of growth, the volume of the cups was adjusted to 200mL with a zoospore suspension to obtain a final concentration of 10$^5$ zoospores/mL of nutrient solution. An equal volume of sterile demineralized water was added to the nutrient solution and served as the control treatment. The cups were wrapped in aluminum foil to avoid the development of algae in the nutrient solution. The cups were arranged in a complete randomized design (CRD) under greenhouse condition where the maximal temperature was 30˚C and 4 replicates were used. One-week later, 100mL of nutrient solution was added to the cup holding the plants to compensate for the water lost by evaporation in order to avoid the water stress. Fifteen days after inoculation, seedlings were removed from the plastics cups and the root system was examined for evidence of discoloration and lesions, and scores were assigned in accordance to the scale of Beneden and Pannecoucque (2008), where 0: no damage; 1: minor discoloration; 2: discoloration plus small necrotic lesion (< 1mm diam); 3: discoloration plus large necrotic lesions (≥ 1mm diam); 4: seedling death (BENEDEN; PANNECOUCQUE, 2008). The disease incidence was estimated as a percentage of plants that presented at least a minor discoloration. Fresh weight of roots and shoots were determined to assess the impact of the disease on the plant growth parameters. The pathogenicity test was done twice.

### 3.4. Zoospores production

*Pythium* zoospores for this study were produced according to the Rahimian and Banihashemi's (1979) method. *Pythium* strains were grown on V8-AC (800 ml of distilled water, 200 ml of 10% V-8 juice, 20 g agar and amended with 2 g of CACO3) plate at 25˚C for 3d. Mycelium plugs were removed from the V8-AC culture with a cork borer and transferred to Petri dishes. The plugs were submitted to a process of washing in order to remove any remaining medium. The plate was first flooded with 20 ml of distilled water in

incubated for 1 h at 25°C. Then, the water was discarded and replaced with 20 of distilled water and the plates were stored at 15°C for 4 days. The water was discarded again in replaced with the same volume and incubated overnight at 15°C to release zoospores. The suspension containing zoospores was passed through a 400 mesh (0.037mm) sterile sieve (A Bronzinox Telas Metálicas e Sintéticas LTDA, Brazil) to remove mycelium and other debris. After sieving, the zoospores density was estimated by vigorously shaking 1 mL of zoospores suspension for 5 min using a vortex. Finally, the encysted zoospores were counted using a hemocytometer (Improved Neubauer, BOECO, Germany) and adjusted to $10^5$ zoospores/ml.

### 3.5. Biosurfactant production

All bacterial strains were grown aerobically in minimal salt medium (MSM) with glucose as the carbon source and ammonium nitrate (NH4NO3) as the nitrogen source (MOUSSA; MOHAMED; SAMAK, 2014, Table 3). A loop of bacterial cells from a 24-h old cultures were inoculated into 200 mL minimal salt medium (MSM) in a 250 mL Erlenmeyer flask. The seed culture was incubated at 37 °C and 200rpm for 7 days, but from the fourth day onwards, samples were taken under sterile conditions to monitor the biosurfactant production using the oil displacement test (data not shown). During the fermentation period the bacterial strains used the glucose to produce the biosurfactant. Foam formation was used as a criterion to monitor the biosurfactant-producing strains (LANGEVIN, 2016) (Figure 3A). After the seventh day, the bacterial culture was transferred to a test tube. The bacteria cells were removed by centrifugation (11000 rpm, 10 min). The cell free supernatant was transferred to a separated tube and screened for biosurfactant production using successively oil dispersing and emulsification index tests. Finally, the tube was stored at 4℃ for further analyses.

Table 3- Composition (g/L) of the minimal salt medium used to produce biosurfactant.

| Components | Quantity (g/L) |
|---|---|
| **Glucose** | 24 |
| **NH4NO3** | 4 |
| **MgSO4.7H2O** | 0.2 |
| **KH2PO4** | 4.08 |
| **Na2HPO4** | 5.68 |
| **Cacl2** | $7\times10^{-4}$ |
| **FeSO4. 7H2O** | $5.56\times10^{-4}$ |
| **Na2EDTA** | $1.5\times10^{-3}$ |

Source: Moussa et al. (2014).

### 3.6. Screening

The oil displacement and emulsification index tests were performed for the detection of bacteria producing biosurfactants. The most promising biosurfactant-producing isolates were selected and tested for lytic activity of zoospore.

### 3.6.1. Oil displacement

For the oil displacement test, 20 ml of distilled water was added to a Petri dish, subsequently 50 µL of soil bean oil was added to surface of the water. Then, 10 µL of cell-free supernatant was added to the surface of the oil (MORIKAWA; HIRATA; IMANAKA, 2000). The presence of biosurfactant in the cell-free supernatant dispersed the oil in the water producing an oil-free clearing zone (Figure 3C). A negative control was performed with sterile distilled water. The oil spreading (OS) was scored as follows: '+', oil spreading with a clear zone ≤ 0.5cm; '++', oil spreading with a clear zone of 0.6 to 2.5 cm; '+++' - oil spreading with a clear zone ≥ 2.6 cm, and "-" indicating the non-dispersion of oil into water (NAYARISSERI; SINGH; SINGH, 2018). The experiment was conducted with three replicates.

### 3.6.2. Emulsification index (E24)

The emulsification activity was determined by adding 5 ml of culture supernatant and 5 ml of kerosene in a 15 ml assay tube. The mixture was vortexed at high speed for 3 min and then kept stand at 15 °C for 24h. Sterile distilled water supplemented with 10% Trypan Blue was used as control (Figure 3B). The E24 index is calculated as the percentage of the height of the emulsified layer divided by the total height of the liquid column according the formula: **E24** = [Height of emulsion formed (cm) /Total height of solution (cm)]*100. The experiment was carried out with three replicates (SHAH et al., 2016).
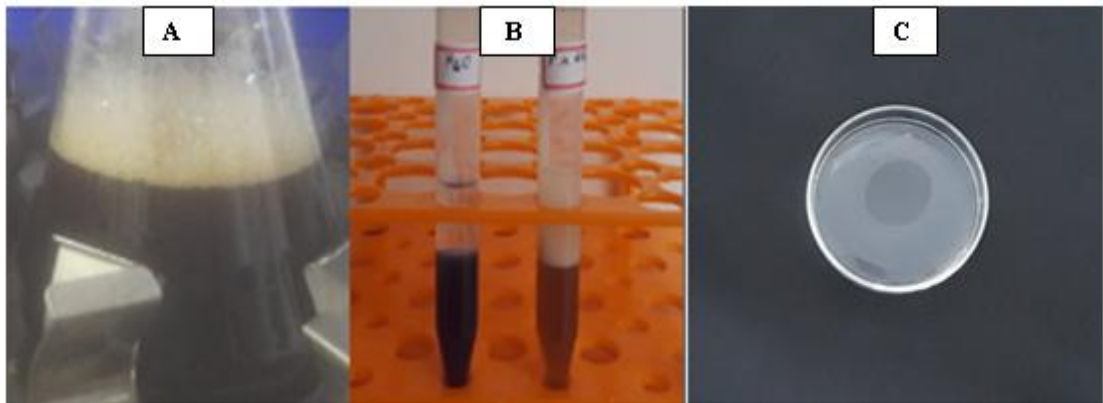
Figure 3- Screening for biosurfactant production in different bacterial strains. A-Foam formation. B-Emulsification. C-Oil displacement test.

## 3.7. Zoospore inhibition assay

The most potent bacterial isolates were further tested for their zoosporicidal activity using a two-fold dilution. For this, 3 mL of zoospores suspension was poured into a test tube. Subsequently, 3 mL of cell-free supernatant was added into the tube to adjust the total volume to 6 mL. A negative control was constituted with 3mL of sterile distilled water was mixed with 3 mL of cell-free supernatant. Samples were taken at 5 min intervals after the treatment to monitor the motility and lysis of zoospores under a light microscope (Carl Zeiss microscopy GmbH, Konigsallee) at 40X. The experiment was performed in triplicate and done twice.

## 3.8. Precipitation of biosurfactants

The biosurfactants were extracted according to the method of  Shah et al. (2016). The cell-free broth of the most promising biosurfactant producer (strain 88A) containing biosurfactant was treated with the mixture of solvent chloroform: methanol: acetone (1:1:1, v: v: v).  The suspension was continuously shaked at 200 rpm, 30°C for 10h to obtain two separate layers. The upper layer was discarded and the precipitated biosurfactant was transferred to plastic pots and frozen at -80°C. The partially purified biosurfactant was lyophilized to evaporate away the solvent, and finally a dry powder containing a crude preparation of the biosurfactants was obtained.

### 3.9. Investigation of the inhibitory activity of the crude biosurfactant

The minimal inhibitory concentration (MIC) was determined by double dilutions. The stock biosurfactant solution was prepared by suspending 100mg of crude biosurfactants in 10 mL of sterile distilled water to obtain a concentration of 10mg/mL. Then, 500 µL of the biosurfactant suspension was directly mixed with 500 µL of zoospores suspension containing $10^5$ zoospores/mL in a 2ml Eppendorf tube to make a final volume of 1mL, sterile demineralized water was used as control. The tube was kept at room temperature. Five minutes later, the lytic activity of biosurfactant containing the culture supernatant was observed under light microscope. Subsequently, the initial solution was diluted to obtain samples with four different concentrations ranging from 1 mg/mL, 2mg/mL, 3 mg/mL and 6mg/mL. Briefly, 500 µL of biosurfactant solution of different concentrations was added into 2mL Eppendorf containing 500 µL of zoospores suspension ($10^5$ zoospores/mL) to obtain a final volume of 1mL with 0.5, 1, 1.5, 3 mg/mL of biosurfactant respectively. The experiment was conducted in triplicate and repeated three times. The inhibitory activity was scored as positive if the zoospores stopped swimming or burst and as negative when the zoospores continued swimming in the suspension.

### 3.10. Genetic characterization of strain 88A
### 3.10.1. DNA extraction, genome sequencing, assembly and annotation

DNA from strain 88A was extracted with Streamlined HMW DNA Purification for Long-Read Sequencing kit (Zymo Research®), according to the manufacturer's recommendations. A genomic DNA sample (~4 µg) was treated with the Rapid Sequencing Kit (SQK-RAD004; Oxford Nanopore Technologies, UK). The resulting library was sequenced by GridION™ platform, using a Spot-ON Mk1 flowcell (FLO-MIN 106, version R9; Oxford Nanopore Technologies) and Library Loading Bead Kit version R9 (EXP-LLB001; Oxford Nanopore Technologies). The raw long reads were obtained with the MinKNOW v3.5.6 program, in a 72-hour run and the base calling was performed simultaneously, using the Albacore v2.0.2 program. The long sequences, obtained from the GridION were assembled by *De novo* approach, using the Canu v1.5 program (KOREN et al., 2017), following the standard parameters for Oxford Nanopore data. Genome annotation was performed by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The genome map of strain 88A was generated using the CGView Server beta online tool (GRANT; STOTHARD, 2008).

### 3.11. Bioinformatics analysis

### 3.11.1. Comparative Genomics

All the genomes fully sequenced of *P. aeruginosa* deposited in the NCBI (National Centre for Biotechnology Information) database as of June 01, 2021 were used in this study. The program BLASTN was used to perform searches for *rhlA*, *rhlB* and *rhlC* genes on all fully sequenced genomes of *P. aeruginosa*. The nucleotide sequences of *rhlA*, *rhlB* and *rhlC* from *P. aeruginosa* 88A were used as queries. Additional searches were performed using the BLASTX and BLASTP programs. For the BLASTP searches, the amino acid sequences of the product of *rhlA, rhlB* and *rhlC* genes were used as queries. All BLAST searches were performed with the default parameters of the programs, except for the filters and masking options that were disabled. The presence and organization of *rhlA*, *rhlB* and *rhlC* genes in *P. aeruginosa* 88A was checked using the web tool SympleSynteny (VELTRI; WIGHT; CROUCH, 2016). The genome of *P. aeruginosa* PAO1 (model strain rhamnolipid production) was used as reference. Additionally, the available metadata for each of the genomes of *P. aeruginosa* (only the completely sequenced ones) deposited at the NCBI were used to register the source and place of isolation of each of the strains used in this study.

The genomic indices Digital DNA–DNA Hybridization (dDDH) and Average Nucleotide Identity (ANI) were calculated using GGDC 2.1 (MEIER-KOLTHOFF et al., 2013) and Kostas Lab (RODRIGUEZ-R; KONSTANTINIDIS, 2014), respectively. The genome sequence of the *P. aeruginosa* 88A and *P. aeruginosa* PAO1 strains (accession numbers: CP074424.1 and NZ_CP053028.1, respectively) were used as reference in this analysis.

### 3.11.2. Phylogenetic analysis

The genome sequence data were uploaded to the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de), for a whole genome-based taxonomic analysis (MEIER-KOLTHOFF; GÖKER, 2019). The TYGS program was used to determine the species of the genus *Pseudomonas* most closely related to *P. aeruginosa* 88A by comparison against all type strain genomes available in the TYGS database. Sequences of the 16S rRNA of the type species most closely related to *P. aeruginosa* were retrieved from NCBI and used in the phylogenetic analyses. The sequences were aligned with MAFFT v7.0 (KATOH; ROZEWICKI; YAMADA, 2018), and phylogenetic analysis with the maximum likelihood method was performed with the program MEGA X (KAVITA; DE METS; GOTTESMAN,

2018). The phylogenomic analyzes were performed using the TYGS server and were conducted using GBDP and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d5 (MEIER-KOLTHOFF et al., 2013). The phylogenetic trees were visualized and edited in the Interactive Tree Of Life (iTOL) v5 Server (LETUNIC; BORK, 2016). In all analyses, 16S rRNA phylogeny and phylogenomics, the genomes of seven strains deposited as *P. aeruginosa* (CMC-115, PA7, A39-1, AZPAE15042, CR1, AR441 and AR_0356), but did not present the *rhlC* gene, were included.

## 3.12. Bioassay

The biological activity of rhamnolipids was evaluated using lettuce (*Lactuca sativa* cv. Roxa) inoculated with *Pythium* zoospores. Eight hundred (800) mL of nutrient solution as described above was added into a 3000 mL container. Two holes of approximately 4.5cm in diameter were drilled in the lid of each container. In each hole, a 50ml plastic cup was added to support the seedlings, resulting in 2 seedlings in each container. After 24h of growth, 200 mL of zoospores suspension was added to each container to make a final volume of 1000mL containing $10^5$ zoospores/mL, an equal volume of sterile demineralized water (SDW) was used as control treatment. The treatments were positive and negative controls, seedlings treated with strain 88A cells ($10^6$/mL) and 88A crude biosurfactant (1mg/mL). The containers were wrapped in aluminum foil. The experiment was conducted in growth chamber at 30 °C and 12 h photoperiod using a complete randomized design (CRD). Each treatment was replicated three times with 2 plants in each replicate, giving 30 plants in the whole experiment. Twenty days after inoculation, seedlings were removed from the containers and the root system was examined for evidence of discoloration, architectural changes and lesions. The disease incidence and severity were determined as described in the section *Pythium* pathogenicity.

## 3.13. Statistical analysis

All measurements are expressed as means ± standard deviation. Data were analyzed using R Studio software. When the normality of the distribution and homogeneity of variances are verified, the analysis of variance (ANOVA) was performed. Statistical analysis was performed at 5% level of significance. The high significant difference (HSD) of Tukey was used to test for significance between means.

## 4. RESULTS

### 4.1. *Pythium* identification and pathogenicity

Sequences of the mitochondrial CoxII gene and the ITS region of the rDNA of strains UFLA 1 and UFLA2 were 100% identical. These sequences were at least 99.8% identical to sequences of the type strain of *Pythium aphanidermatum* and to sequences of the strain DAOM BR444, used as a representative to sequence the genome of this species (Table 4). In contrast, sequences of the ITS and CoxII were 97.5 and 96.9% identical to sequences of the type strain of *P. deliense*, which was the second closest match in Blast searches (Table 4). These results clearly show that strains UFLA1 and UFLA2 belong in the species *P. aphanidermatum*.

Strains UFLA1 and UFLA2 were tested for their ability to induce diseases in lettuce in hydroponics. The disease severity varied from 2.5 to 2.75 between the isolates, which was significantly different from the treatment without *Pythium*. Inoculated plants showed a significant growth reduction of shoots and roots fresh weight in comparison with the control (Figure 4). The percentage of reduction in root fresh weight was on average 41% and shoot fresh weight was 42.5% (Table 5).

Table 4- Identity between sequences from the strains identified in this study (UFLA1 and 2) and sequences from strains in public databases. *Pythium deliense* is one of the closest to *P. aphanidermatum.*

| Species/ strains | ITS [b] | CoxII [b] |
|---|---|---|
| *P. aphanidermatum* **UFLA1 and UFLA2** | (863 bp) | (613 bp) |
| *P. aphanidermatum* **CBS 118.80[T]** | **99.77%** (863 bp) [AY598622.2] | **99.80%** (547 bp) [KJ595344.1] |
| *P. aphanidermatum* **DAOM BR444** [a] | **100%** (672 bp) Scaffold_1086 [KE464950.1] | **99.84%** (613 bp) scaffold_784 [KE464648.1] |
| *P. deliense* **CBS 314.33[T]** | **97.45%** (863 bp) [AY595372.2] | **96.94%** (556 bp) [KJ595372.1] |

[T] type strain; [a] Unfinished genome; [b] size of the DNA fragment compared between parentheses; identity (%) in comparisons between the strains used in this study (UFLA1 and 2) and sequences from public databases; accession numbers between square brackets.

Table 5- Pathogenicity of *Pythium aphanidermatum* on hydroponically grown lettuce.

| Treatment | Incidence | Severity | Fresh root weight (g/plant) | Fresh shoot weight (g/plant) |
|---|---|---|---|---|
| Control | 0.00 | $0.00 \pm 0.00$ a | $2.77 \pm 0.51$ b | $9.91 \pm 2.06$ b |
| UFLA 1 | 100.00 | $2.50 \pm 0.58$ b | $1.65 \pm 0.14$ a | $6.05 \pm 1.11$ a |
| UFLA 2 | 100.00 | $2.75 \pm 0.50$ b | $1.63 \pm 0.14$ a | $5.37 \pm 1.20$ a |

Means with different letter in each column are significantly different (Tukey, 0.05), (n=12).

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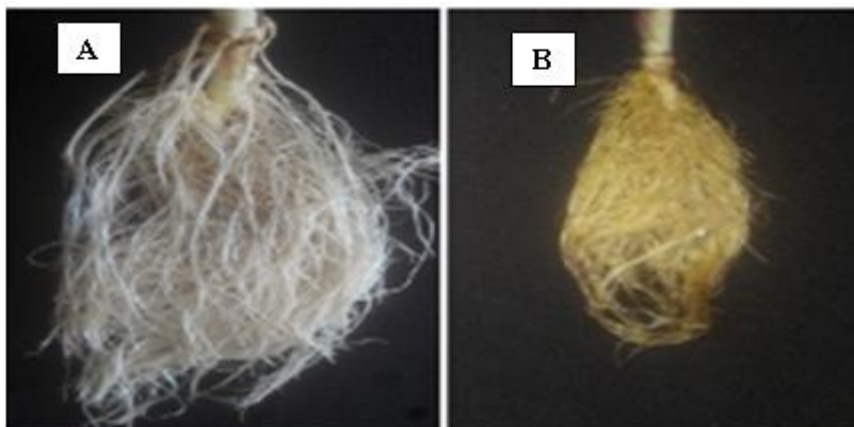
Figure 4- Pathogenicity of *P. aphanidermatum*. A- non-inoculated lettuce roots. B- Lettuce roots inoculated with *Pythium*.

## 4.2. Screening for biosurfactant production

In this study, six bacterial strains (LBB50, BINV, BMH, LBB47, LBB58 and 88A) were screened for biosurfactant production. The biosurfactant production was monitored on the basis of foaming. Foaming began from the third day of incubation with continuous shaking at 37∘C, while excessive foaming was observed after the seventh day with strain 88A, followed by strains LBB50 and BMH, whereas strain BINV did not produce any foaming. The emulsifying activity was determined by measuring the emulsion height after 24h (E24) using kerosene. The E24 of the biosurfactant containing cell-free supernatant varied from 0% for strain BINV to 45.45 % for strain 88A (Table 6). Biosurfactant activity was also monitored using oil spreading test (OST). The results showed that, 10 µL of cell-free supernatant produced an oil free clearing zone with diameter ranging from 0.5-2.6 cm for BMH in values equal or higher than 2.6 cm for strains LBB50 and 88A (Table 6).

The most promising biosurfactant-producing bacterial strains were further tested for their inhibitory activities against *P. aphanidermatum* zoospores. The biosurfactant containing cell-free supernatants of strain 88A halted the motility of zoospores in approximately 5 min after the treatment (Table 7), while 10 min were needed to observe the halting with strain LBB50 and BMH. The cell-cell free supernatant containing surfactants of strain 88A displayed a stronger inhibitory activity when compared with strains LBB50 and BmH. The zoospores were lysed in 10 and 15 min after treatment with strains 88A and LBB50 respectively. However, the supernatant of strain BmH was unable to lyse zoospores (Table 7).

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Zoospores in the control (no culture supernatant) were still swimming 15 min after the treatment. The zoospores that stopped swimming due to the adverse effect of biosurfactants present in the suspension became round or lysed (Figures 5). Strain 88A showed the strongest surfactants activity and was selected for genetic characterization and activity *in planta.*

Table 6- Screening of bacterial strains for biosurfactant production based on E24 (%) and mean diameter of oil spreading. All values are means ± SD for triplicates sample.

| Bacterial strains | E-24 | Oil spreading (OS) |
|---|---|---|
| Control-distilled water | $0.00 \pm 0.00$ | - |
| LBB50 | $40.25 \pm 3.30$ | +++ |
| BmH | $34.14 \pm 1.96$ | ++ |
| BINV | $0.00 \pm 0.00$ | - |
| 88A | $45.45 \pm 2.35$ | +++ |
| LBB47 | $4.55 \pm 0.96$ | - |
| LBB58 | $5.48 \pm 0.91$ | - |

Table 7- Effect of biosurfactant containing culture supernatant of strains LBB50, BMH and 88A on *Pythium* zoospores.

| Treatments | 5 min | | 10 min | | 15 min | |
|---|---|---|---|---|---|---|
| | Halted | Burst | Halted | Burst | Halted | Burst |
| SDW | - | - | - | - | - | - |
| LBB50 | - | - | + | - | + | + |
| BMH | - | - | + | - | + | - |
| 88A | + | - | + | + | + | + |

**SDW:** Sterile demineralized water
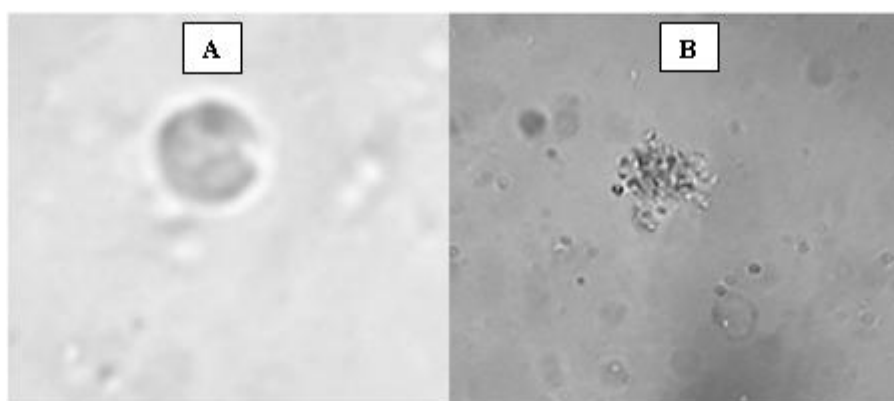


Figure 5- Inhibition of zoospores activity. A-Zoospores in round shape zoospores 5 min after treatment with culture supernatant of strain 88A. B-Zoospores lysis 10 min after treatment with the culture supernatant of strain 88A.

## 4.3. Characterization of strain 88A: genomics, phylogeny and comparative genomics

The genome of strain 88A was sequenced, assembled and annotated and the genome features indicated that it was a typical *Pseudomonas aeruginosa* (Table 8).

Table 8- Summary of general genomic features of *Pseudomonas aeruginosa* 88A.

| Genome Features | *Pseudomonas aeruginosa* 88A |
| --- | --- |
| Genome size (pb) | 6,378,355 |
| Genome CDS count | 5,848 |
| Contig | 1 |
| 5S + 16S + 23S rRNA count | 4 + 4 + 4 |
| tRNA count | 64 |
| GC content (%) | 66.49 |
| GenBank acession number | CP074424.1 |



**Pseudomonas aeruginosa 88A**
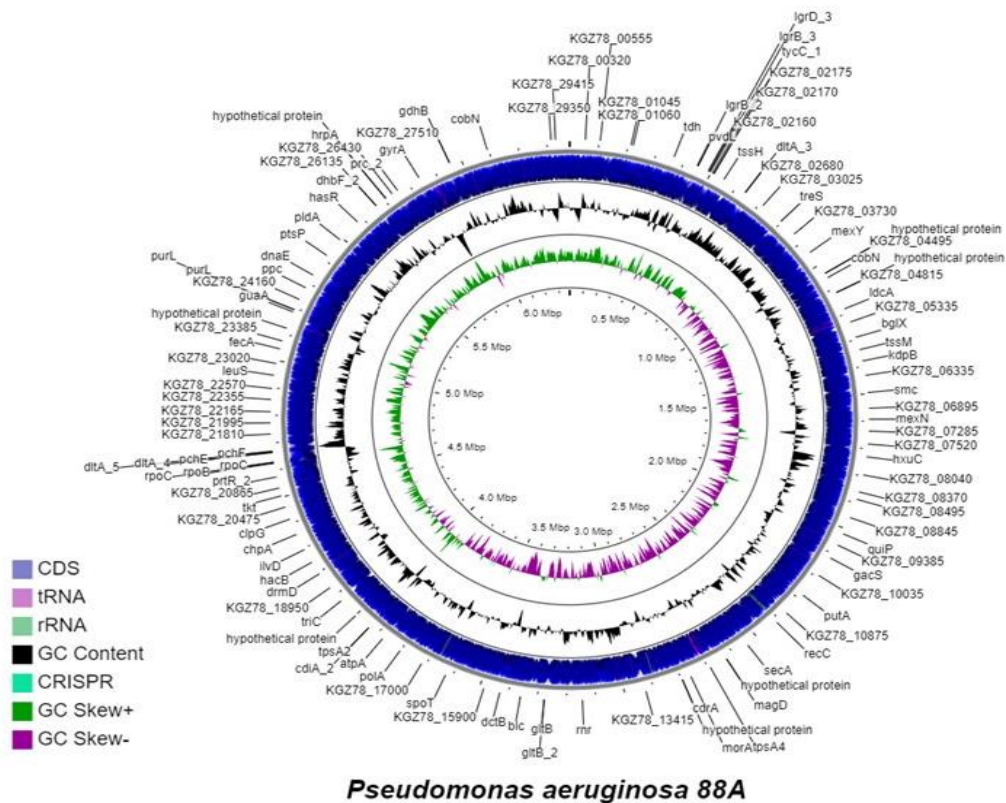
Figure 6- Circular chromosome map of *Pseudomonas aeruginosa* strain 88A showing the distribution of coding sequences (CDS), tRNAs, rRNAs, GC content skew (50% of the total base pair window), and CRISPR. The map was generated using the CGView Server beta online tool.

From the 309 genomes included in this study, 302 were identified as *Pseudomonas aeruginosa,* whereas strain CMC-115 was classified as a putative subspecies of *Pseudomonas aeruginosa* and 6 strains as a putative new species of the genus *Pseudomonas* on the basis of genomic indices (Table 9). These results were also confirmed with a phylogenetic analysis with the 16S gene and with a phylogenomic analysis of the whole genome of representative strains of *P. aeruginosa* (Figure 7).

Table 9- Phylogenomic parameters calculated for *P. aeruginosa* strains that do not shown in their genomes at least one of the essential genes related to the production of rhamnolipids (*rhlA*, *rhlB* and *rhlC*). Genomic indices (ANI and dDDH) were calculated in relation to the genomes of PAO1 and 88A strains.

| *P. aeruginosa* strains | Accession numbers | Current classification | PAO1$^T$ | | 88A | | Putative Reclassification |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | | ANI (%)$^*$ | dDDH (%)$^*$ | ANI (%)* | dDDH (%)* | |
| PAO1 | NZ_CP053028.1 | *P. aeruginosa* | - | - | 99.25 | 93.80 | - |
| 88A | CP074424.1 | *P. aeruginosa* | 99.25 | 93.80 | **-** | **-** | **-** |
| CMC-115 | NZ_CP046602.1 | *P. aeruginosa* | 97.42 | 78.20 | 97.33 | 77.90 | **Subspecies** |
| PA7 | NC_009656.1 | *P. aeruginosa* | **93.27** | **52.70** | **93.26** | **52.90** | **New species** |
| A39-1 | NZ_CP068238.1 | *P. aeruginosa* | **93.12** | **52.00** | **93.04** | **52.20** | **New species** |
| AZPAE15042 | NZ_CP041354.1 | *P. aeruginosa* | **93.06** | **51.80** | **93.07** | **52.10** | **New species** |
| CR1 | NZ_CP020560.1 | *P. aeruginosa* | **93.07** | **51.80** | **93.04** | **52.10** | **New species** |
| AR441 | NZ_CP029093.1 | *P. aeruginosa* | **93.16** | **51.80** | **93.03** | **52.10** | **New species** |
| AR_0356 | NZ_CP027169.1 | *P. aeruginosa* | **93.18** | **51.80** | **93.09** | **52.10** | **New species** |

Average Nucleotide Acid Identity (ANI) and Digital DNA-DNA Hybridization (dDDH) comparisons with the *P. aeruginosa* PAO1 (Ramnolipid production model strain) and *P. aerginosa* 88A (strain used in this study). Values out of the range for species delineation are shown in bold (ANI> 95%, Auch et al. 2010; dDDH> 70%, Richter et al. 2015). The genomes of strains CMC-115, PA7, A39-1, AZPAE15042, CR1, AR441 and AR_0356 were investigated because no copy of the *rhlC* gene was found.
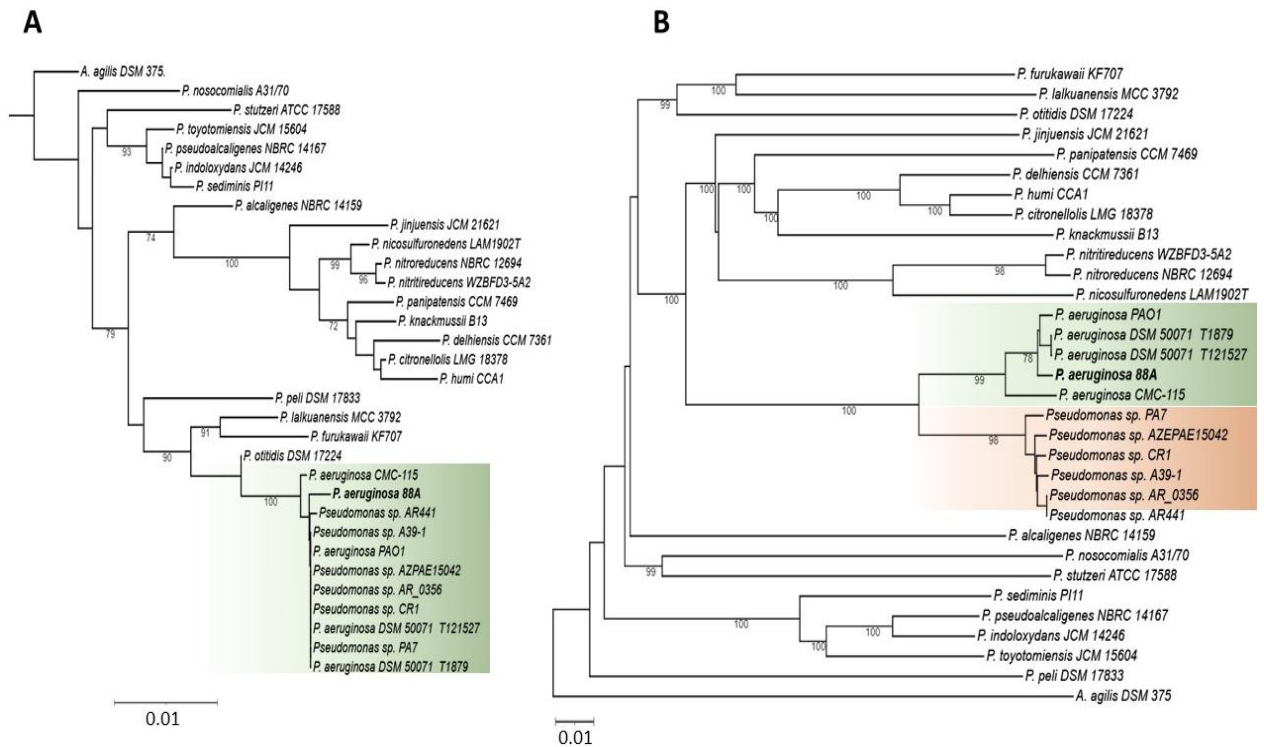
Figure 7- Phylogenetic tree with the taxonomic placement of strain 88A and other *Pseudomonas* species. (A) Phylogenetic tree constructed with the maximum likelihood method with 16S rRNA gene sequences of *Pseudomonas*. The tree was generated with 1,533 bp-aligned nucleotides of the *P. aeruginosa* 88A and other sequences from species type from the LPSN webpage (List of Prokaryotic Names with Standing in Nomenclature). The phylogenetic analysis was performed the GTR+G substitution model and 1,000 bootstrap resamplings. Numbers above branches indicate bootstrap support, and the tree was rooted with sequences of *Azomonas agilis* DSM 375. The scale represents the number of expected substitutions per site. (B) The phylogenomic tree was performed with the closest type strains genomes of *P. aeruginosa*, including the 88A strain, seven genomes from the strains that not shown *rhlC* gene copies and the *A. agilis* DSM 375 genome. The genome sequences were uploaded to the Type (Strain) Genome Server - TYGS (https://tygs.dsmz.de) for a whole genome-based taxonomic analysis (MEIER-KOLTHOFF; GÖKER, 2019). For each genome analyzed, the precise distance was calculated using the Genome BLAST Distance Phylogeny approach (GBDP) by the FastME 2.1.6.1 program (Lefort et al., 2015). The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 77.2 %. Bootstrap values above 70% are shown. The tree was rooted with the genome sequence of *A. agilis* DSM 375.

## 4.4. Geographic distribution and isolation sources of the 303 *Pseudomonas aeruginosa* strains with fully sequenced genomes deposited in public databases.

The 303 genomes of *P. aeruginosa* (including strain 88A and the putative subspecies, strain CNC-115) were isolated from all over the world, but mostly from China and the US (Figure 8). The great majority (~85%) of the strains were obtained from humans and other animals, and the rest from diverse environments, such as soils, plants and industrial settings.
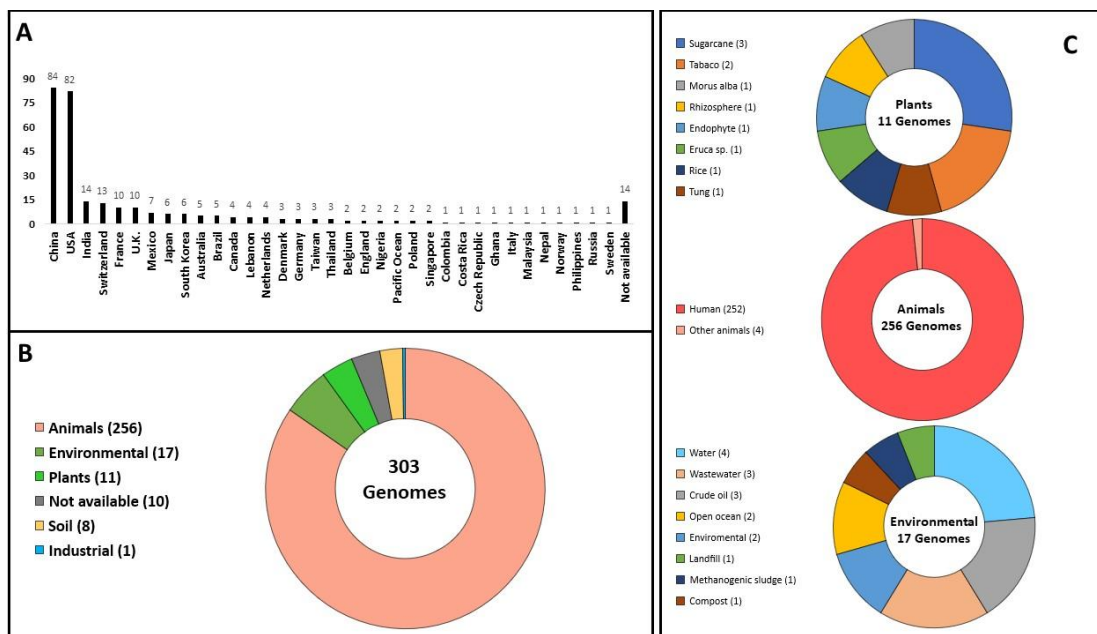
Figure 8- Geographic distribution and isolation sources of the 303 *Pseudomonas aeruginosa* strains with completely sequenced genomes deposited in GenBank (NCBI). (A) Isolation site (countries) of *P. aeruginosa* strains. (B) Isolation source of *P. aeruginosa* strains with completely sequenced genome. (C) Categorization and description of different isolation sources of *P. aeruginosa* strains.

## 4.4. Diversity in distribution of biosynthetic rhamnolipid genes in sequenced genomes

The identity between rhamnolipid genes was highest among strains classified as *Pseudomonas aeruginosa* when compared with the putative subspecies and the putative novel species (Tables 9 and 10). The gene *rhlC* was slightly more diverse than *rhlA* and *rhlB* of *P. aeruginosa* (Table 10).

All the 309 genomes harbored *rhlAB genes,* but 6 genomes did not have the genes *rhlC* (Table 9). All genomes had one copy of the *rhl* genes, except for the genome of strain SP4527, which had two copies for each of the three genes (Table 10). These analyses confirmed that all known strains of *P. aeruginosa* harbor genes for rhamnolipid production. These genes have a similar organization in strain 88A and PAO1, except that the order of *rhl*C gene is inversed in these two strains (Figure 9).

Table 10- Diversity of rhamnolipid genes in genome of strains deposited as *P. aeruginosa* in public databases. All comparisons were done with strain 88A and therefore comparisons with itself were not performed.

| Complete Genomes | Identity in relation to *P. aeruginosa* 88A | | |
|---|---|---|---|
| | *rhlA* (%) | *rhlB* (%) | *rhlC* (%) |
| *P. aeruginosa* (300 genomes) | 98.99-99.89 | 98.71-100.00 | 97.04-100.00 |
| *P. aeruginosa* SP4527 | 99.44 (2*) | 99.14 (2*) | 99.80 (2*) |
| *P. aeruginosa* CMC-115 | 95.50 | 97.00 | nf** |
| *Pseudomonas* sp.(6genomes) | 91.68-92.13 | 91.28-91.70 | nf** |

* Number of copies of *rhlA*, *rhlB* and *rhlC* genes found in the genome of *P. aeruginosa* strain SP4527. The identity value of the additional genes copies was the same in relation to *P. aeruginosa* 88A.
** Not found.
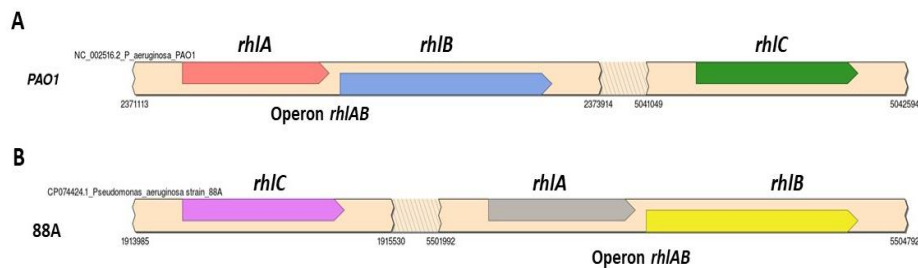


Figure 9- Presence and organization of genes involved in rhamnolipid production in *Pseudomonas aeruginosa.* (A) Synteny of the *rhlAB* operon and the *rhlC* gene, involved in the production of rhamnolipids in *P. aeruginosa* PAO1 (model strain for the production of rhamnolipids in *P. aeruginosa*). (B) Presence and synteny of genes involved in rhamnolipid production in *P. aeruginosa* 88A.

## 4.5. Activity of rhamnolipids from strain 88A against *Pythium*

The amount of rhamnolipids recovered after growing strains in glucose as the carbon source and ammonium nitrate as nitrogen source was 2.75 g/L. The dry crude rhamnolipid (figure 10) recovered from 88A strain was tested for zoosporicidal activity against *Pythium* zoospores. The crude rhamnolipid produced by strain 88A lysed completely the zoospores at a minimum concentration of 1mg/mL after approximately 5 min of treatment (Table 11).

The applicability of strain 88A or the crude rhamnolipids was evaluated by examining their effects on *Pythium* zoospores on lettuce grown in hydroponics. Strain 88A

Table 11- Minimum inhibitory concentration (MIC) of precipitated rhamnolipids of strain 88A.

| Concentration | Lysis |
|---|---|
| 3 mg/ml | + |
| 1.5 mg/mL | + |
| 1 mg/mL | + |
| 0.5 mg/mL | - |
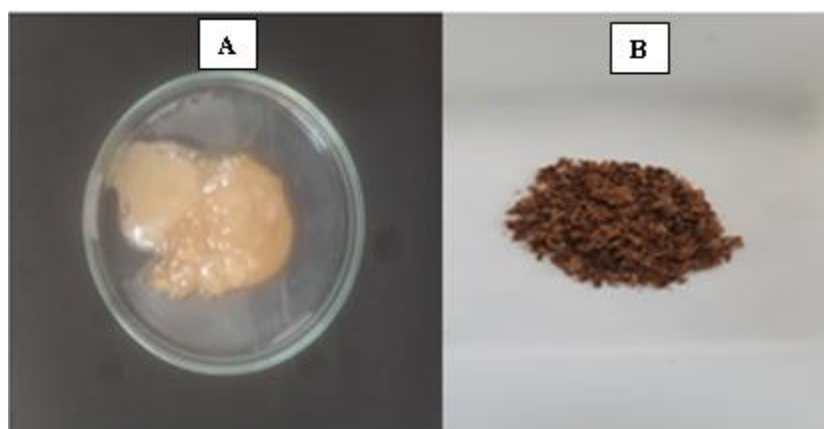


Figure 10- Recovery of biosurfactants. A- Precipitated biosurfactant after fermentation. B- Brownishdry biosurfactants after lyophilizing.

Table 12- Effect of *Pseudomonas aeruginosa* 88A and its precipitated rhamnolipids against *Pythium*-zoospores in a soilless lettuce.

| Treatment | Severity | Fresh root weight (g/plant) | Fresh shoot weight (g/plant) |
|---|---|---|---|
| Non-inoculated control | $0.00 \pm 0.00$ c | $1.32 \pm 0.17$ a | $13.67 \pm 0.4$ a |
| Inoculated control | $3.00 \pm 0.00$ a | $0.54 \pm 0.01$ b | $3.78 \pm 0.38$ b |
| *P.aeruginosa* 88A-Cells | $1.17 \pm 0.29$ b | $1.28 \pm 0.19$ a | $12.17 \pm 1.32$ a |
| *P.aeruginosa* 88A-surfactant | $1.17 \pm 0.29$ b | $1.30 \pm 0.04$ a | $12.10 \pm 0.61$ a |

Means with different letter in each column, are significantly different (Tukey, 0.05), (n=12).

Figure 11- Illustration of the biological experiment. A- Un-inoculated plant vs. inoculated plant. B-Inoculated plant treated with rhamnolipid vs. non-treated plant.

cells or its crude rhamnolipids biosurfactant significantly ($P<0.05$) suppressed disease severity. The mean reduction in severity was $61\pm 0.14$ %. Plants treated with strain 88A or the crude rhamnolipids exhibited significantly improved growth when compared with the control inoculated with *Pythium*. The fresh weight of roots increased significantly from 0.54 in plants inoculated with *Pythium* only to approximately 1.28 g/plant system, which was not significantly different from the treatment without the pathogen (Table 12). Similar results were found for the aerial part of the lettuce. Conversely, plants treated with the culture supernatant died two days after treatments (data not shown), which may be due to a phytotoxicity created by the high nitrogen concentration contained in the suspension.

## 5. DISCUSSION

*Pythium* zoospores are the main source of inoculum in hydroponics. Managing this asexual stage reduces disease incidence and severity (ISLAM; LAATSCH; TIEDEMANN, 2016). *Pythium* causes serious damage in hydroponic lettuce all over the world (CORRÊA; BETTIOL; SUTTON, 2010; STANGHELLINI; MILLER, 1997). *Pythium* species infect the roots and interfere with plant development (DA SILVA et al., 2019; RAUDALES; MCGHEE, 2016). *Pythium aphanidermatum* is one of the most commonly reported species in hydroponic lettuce (CORRÊA; BETTIOL; SUTTON, 2010; DA SILVA et al., 2019).

In this study, two *Pythium* strains were identified as *P. aphanidermatum* by sequencing analysis and their pathogenicity in lettuce was demonstrated. The induced symptoms included root discoloration, brown lesions on root tips and root rot. Infected plants showed decreased production of fresh matter. The infected plants with strains UFLA1 and UFLA2 showed symptoms typical of those induced by *Pythium aphanidermatum* (DA SILVA PATEKOSKI; PIRES-ZOTTARELLI, 2010; RAUDALES; MCGHEE, 2016). *Pythium aphanidermatum* is a species known to cause problems in dicotyledonous plants grown in high temperatures (LÉVESQUE; DE COCK, 2004). The experiments reported here were performed at temperatures around 30 ºC, indicating that this species prefers high temperatures. At fluctuating temperatures of ~20-28 ºC during the day and ~15 ºC at night there was no disease development (data not shown).

Some of the bacterial strains used in the screening experiments were known to produce surfactants, but we did not know their chemical identity. The screening methods used in this study have the advantages of being simple, not requiring specialized equipment, inexpensive and of quick implementation. The disadvantage of these methods is that they do not allow the identification of the compounds. In this study, we did not present any direct proof for the identity of these surfactants. Our indirect evidence comes from the fact that strain 88A was identified by several methods as *P. aeruginosa* and our bioinformatic analysis confirmed that all known strains of *P. aeruginosa* harbor genes for the synthesis of rhamnolipids. Therefore, we are confident that the compounds present in the culture supernatant of strain 88A and in the precipitated powder produced thereafter and for which we have shown compelling evidence of their zoosporicidal activity and ability to control *P. aphanidermatum* in lettuce are rhamnolipids. Other authors have shown similar results with

surfactants produced by *P. aeruginosa* against zoospores of *Pythium* and *Phytopthtora* ((DEEPIKA; RAMU SRIDHAR; BRAMHACHARI, 2015; ZOHARA et al., 2016). Besides rhamnolipids, *Pseudomonas aeruginosa* can produce others compounds with antagonistic activities against plants pathogen (MORUZZI et al., 2017; PERNEEL et al., 2008). Furthermore, these are the only surfactants produced by *P. aeruginosa* that are able to cause zoospore lysis (PERNEEL et al., 2008).

Most of the organisms are highly dependent on the medium and temperature for growth and production of secondary metabolites. Rhamnolipid production can be enhanced when growth temperature and pH values are carefully controlled. The results in the present study revealed that *P. aeruginosa* strain 88A can produce rhamnolipids in minimal salts medium at 37∘C, which correlates with several previous investigations (DEEPIKA; RAMU SRIDHAR; BRAMHACHARI, 2015; OCHSNER; HEMBACH; FIECHTER, 1996; SHAH et al., 2016; SIDKEY; MOHAMED; ELKHOULY, 2016). Over the past ten years, several investigations have been carried out to understand the mechanism behind the formation of rhamnolipids (WITTGENS et al., 2017). These studies revealed that the biosynthesis of rhamnolipids is regulated by quorum sensing and directed by three *rhl* genes (A,B,C) (TAN; LI, 2018; THAKUR et al., 2021). Our genomic analyses have shown that the organization of these genes may vary in different strains of *P. aeruginosa*, but one hallmark of this species is the presence of the three biosynthetic genes (*rhl*AB and *rhl*C) in all the 303 fully sequenced genomes of this species deposited in public databases.

The rhamnolipids from strain 88A were partially purified with a solvent mixture of chloroform-methanol-acetone and lyophilized. The yield of this crude preparation produced by strain 88A was estimated to be 2.75 g/L. Similar yields were previously reported (PATIL; PENDSE; ARUNA, 2014). Patil et al. (2014) reported a yield of 2.8 g/L for *P. aeruginisa* F23, while a lower yield (1.7 g/L) was reported for *P. aeruginosa* strain R (KARKERA et al. 2012). In this study we did not attempt any optimization, but possibly, higher yields may be obtained with methodologies such surface response and alternative substrates.

The MIC values confirmed the zoosporicidal activity of strain 88A rhamnolipids. These compounds are involved in the lysis of the plasma membrane of zoospores (ISLAM; LAATSCH; TIEDEMANN, 2016; SHARMA et al., 2007). Similar observations were done by Yoo et al. (2005) and Stanghelini and Miller (1997).

The efficacy of strain 88A and its derived rhamnolipid biosurfactant was evaluated in a biological experiment. Strain 88A and its rhamnolipids decreased significantly the disease severity. There is a great deal of information available on the biological control of plants diseases by the strains of *Pseudomonas* (DE JONGHE et al., 2005; TRAN; KRUIJT; RAAIJMAKERS, 2008). Over the past 10 years, the significance role of rhamnolipid biosurfactant in disease control and plant growth promotion have been elucidated (CHOPRA et al., 2020; DEEPIKA; RAMU SRIDHAR; BRAMHACHARI, 2015; MISHRA et al., 2020). Results obtained in this study showed that cells or rhamnolipids from *P. aeruginosa* strain 88A reduced disease severity on hydroponically grown lettuce artificially infected with *Pythium* zoospores. Results in accordance with this study were obtained previously from testing antagonistic strains of *P. aeruginosa* (DE JONGHE et al., 2005; SHARMA et al., 2007; ZOHARA et al., 2016).

Rhamnolipids are eco-friendly because they are rapidly biodegraded, in contrast with chemically-produced surfactants. Synthetic surfactants were previously applied in the control of *Pythium* in hydroponics (BAGNALL, 2008; STANGHELLINI, Michael E.; MILLER, 1997) and were able to disintegrate the plasma membrane of zoospores, resulting in a loss of the motility and quick lysis. However, these chemical surfactants have little or no effect in the other pathogen life stages (STANGHELLINI, Michael E.; MILLER, 1997). Conversely, rhamnolipids exhibited potential in inhibition of sporangia formation, zoospore encystment and hyphal growth of several *Pythium* species (SHARMA et al., 2007; STANGHELLINI; MILLER, 1997). Yoo et al. (2005) reported the efficacy of rhamnolipids in the reduction of damping off caused by *Pythium*. Due to their low biodegradability, synthetic surfactants generates the toxic substance in the nutrient solution, which is detrimental for plant growth (BAGNALL, 2008).

From the 303 fully sequenced genomes of *P. aeruginosa*, only 3.6 and 2.6% of the strains were isolated from plants and soil, respectively. These results indicate that *P. aeruginosa* is a well-known bacterial pathogen associated with human patients (CROSS et al., 1983; KERR; SNELLING, 2009). However, these data may be biased because more resources are invested in human diseases than in environmental studies. Almost certainly, if more environmental samples are collected, more *P. aeruginosa* will be found. This range of

distribution that we verified in our analyses emphasizes the importance of *P. aeruginosa* as an opportunistic species.

Unfortunately, due to its capacity to cause diseases in humans, it is unlikely that regulatory agencies will approve its use as living cells in agricultural crops. Companies such as Marrone Bioinnovations Inc. are already producing cell-free supernatants of *Burkholderia*, another bacterium that may cause problems in immunocompromised humans, to control plant pathogens. The application of the crude preparations of rhamnolipids is effective in the control of *P. aphanidermatum* in lettuce grown hydroponically. Although this practice cannot be considered as biological control, it may be a feasible and eco-friendly strategy. Further studies should be done to assess the economic viability of these compounds in commercial farms.

## 5. CONCLUSION

Results of this study showed that *P. aeruginosa* 88A, BMH and LBB50 were able to produce biosurfactants with inhibitory effects against *Pythium* zoospores. Additionally, we found that the strain *P. Aeruginosa* 88A generates biosurfactant with highest inhibitory activities. Results from a biological experiment demonstrated that strain 88A or the precipitated rhamnolipids decreased *Pythium* severity in hydroponic lettuce by approximately 60% and increased fresh weight of lettuce plants by 68%, which was not significantly different from the treatment without *Pythium*. Although this bacterial species is frequently associated with immunocompromised patients, the purified rhamnolipids may be applied in the control of *Pythium* in hydroponic lettuce.

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